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C12N 5/00

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(54) Culture medium for mammalian embryos

(57) A culture medium for mammalian embryos *in vitro* comprises at least one iron chelator for example bovine apotransferrin, ethylenediaminetetracetic acid (EDTA) or diethylenetriaminepentaacetic acid (DETAPAC). The culture medium is particularly useful in a method of *in vitro* mammalian embryo culture comprising culturing an embryo in the culture medium at least in the period between fertilisation and the formation of a blastocyst.

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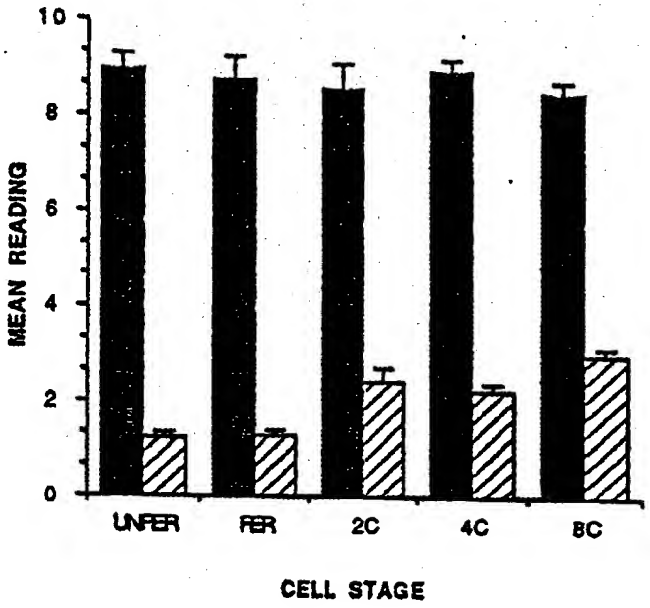
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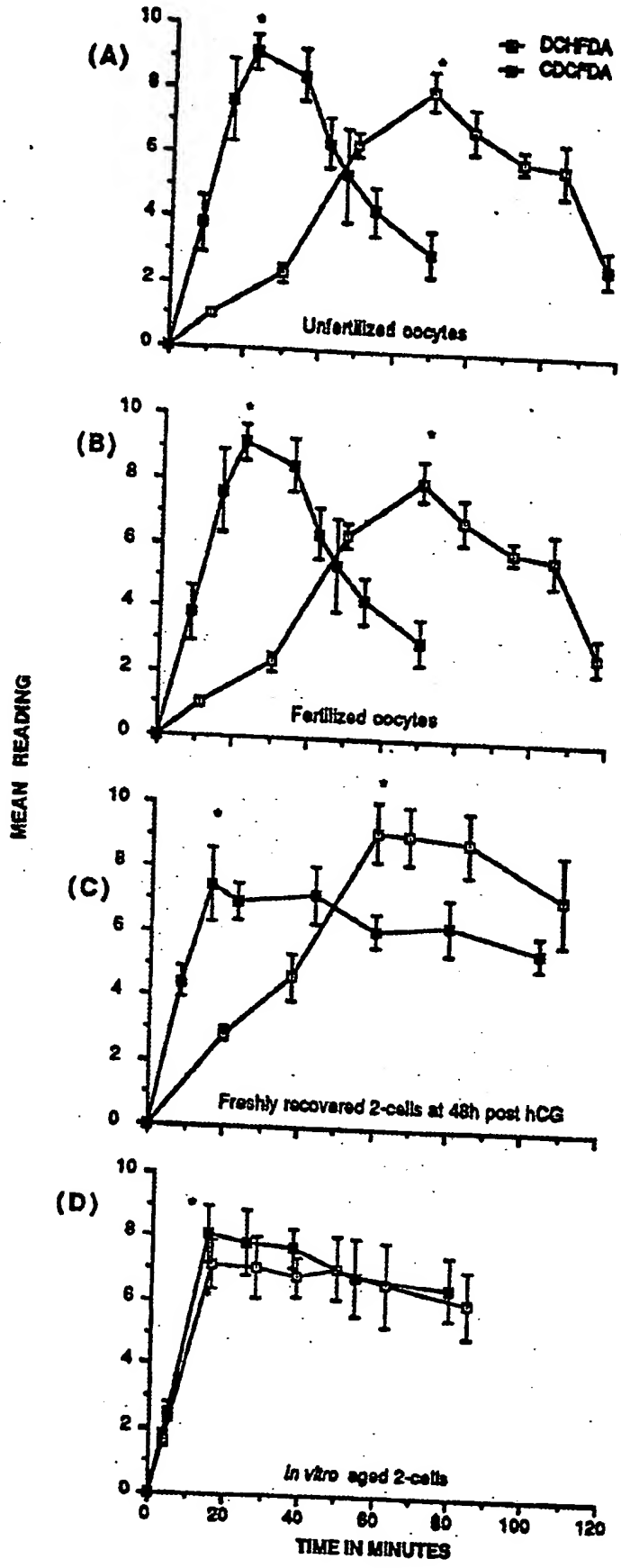
CELL STAGE


(A)
DCHFDA
CDCFDA
Unfertilized oocytes
(B)
Fertilized oocytes
(C)
Freshly recovered 2-cells at 48h post hCG
(D)
In vitro aged 2-cells
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2
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6
8
10
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80
100
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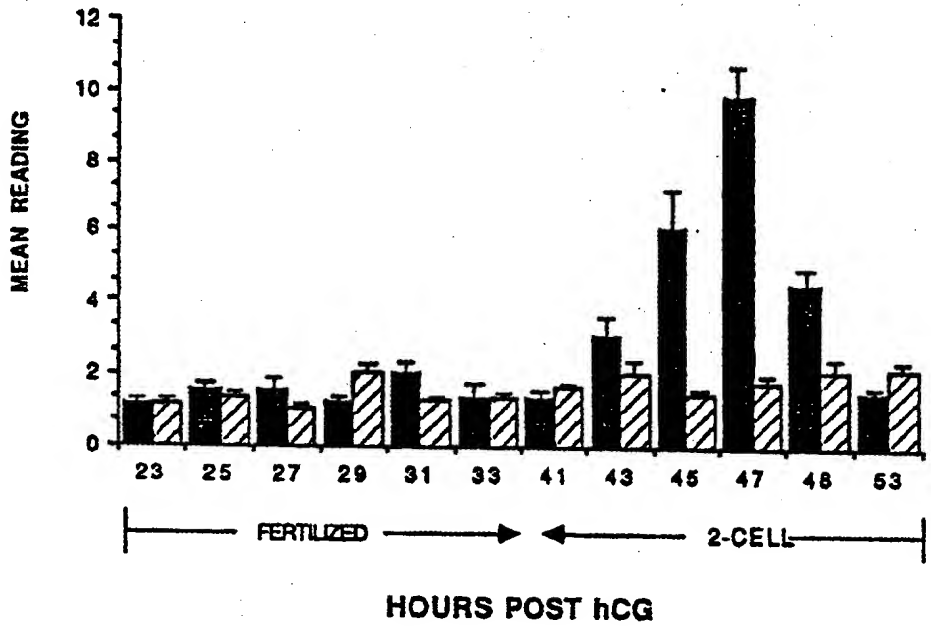
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8
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4
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53
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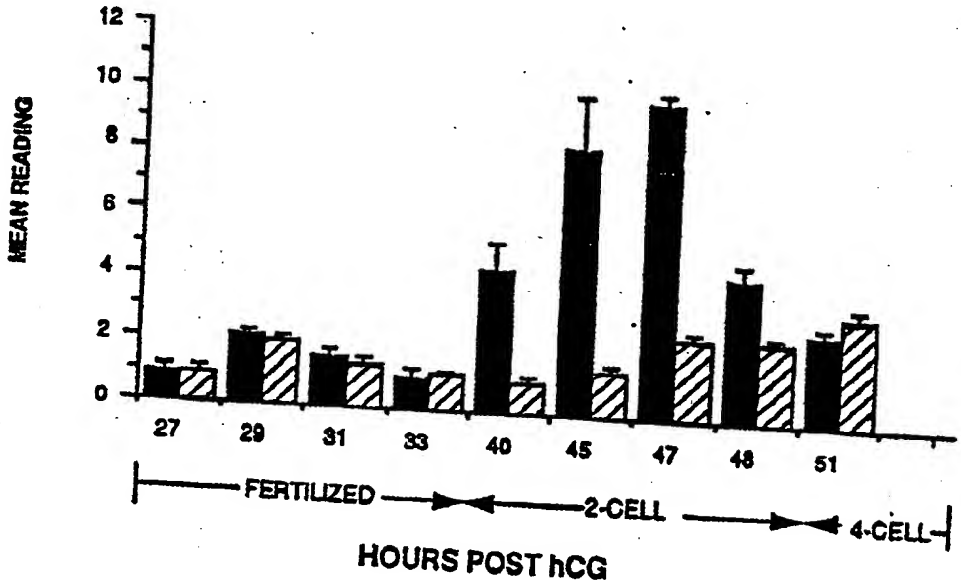
2-CELL
HOURS POST hCG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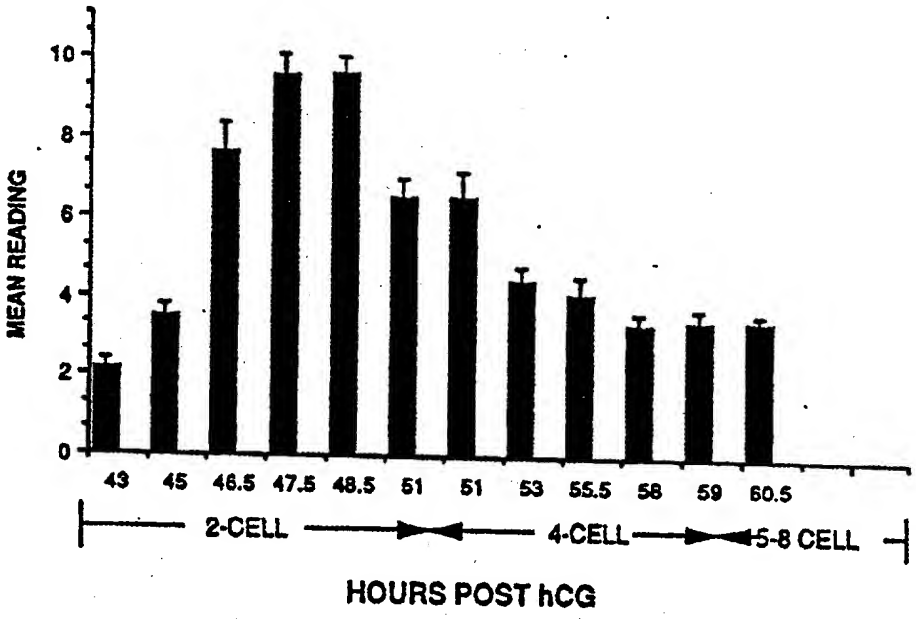
FERTILIZED
2-CELL
4-CELL
HOURS POST hCG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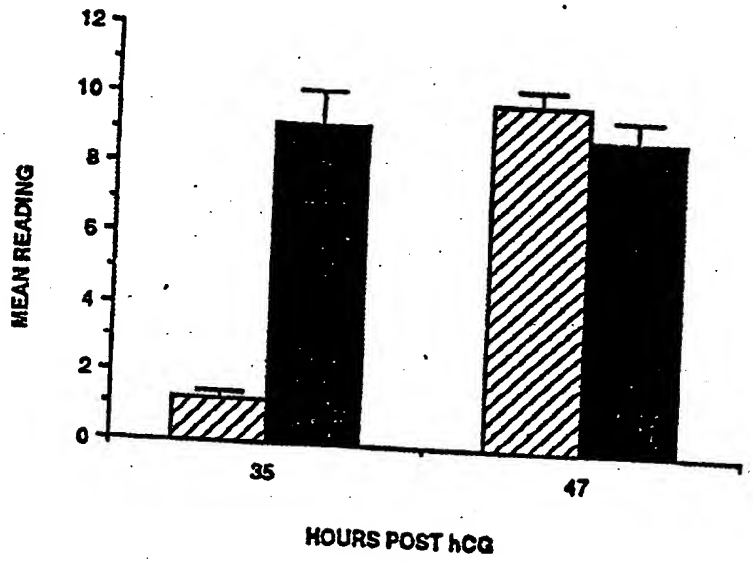
5-8 CELL
HOURS POST hCG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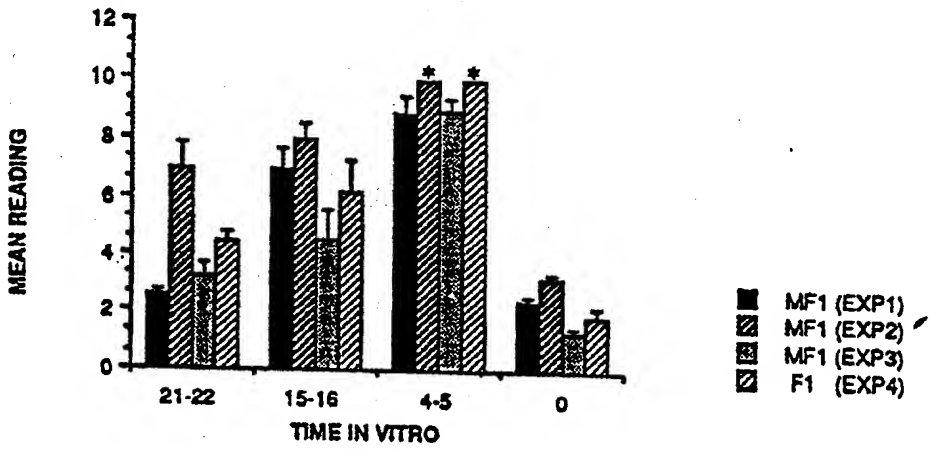
6
4
2
0
MEAN READING
35
47
HOURS POST hCG


MEAN READING
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8
6
4
2
0
21-22
15-16
4-5
0
TIME IN VITRO
MF1 (EXP1)
MF1 (EXP2)
MF1 (EXP3)
F1 (EXP4)

# CULTURE MEDIUM

This invention relates to a culture medium for mammalian embryos *in vitro*. In particular, it relates to a medium for the culture of embryos in the crucial period between fertilisation and the blastocyst stage and is of use in, for example, *in vitro* fertilisation techniques.

For most mammalian species, it has not proved possible to culture embryos from fertilization through to the blastocyst stage. Embryos arrest at a stage characteristic for the species.

In all species studied, evidence has been found for arrest of development at a specific cell stage, e.g. mouse 2-cell (Whittingham, 1974; Goddard & Pratt, 1983), human 4-8 cell (Braude, Bolton & Moore, 1988), hamster 2-cell (Carney & Bavister, 1987; Bavister, 1988), sheep and goat 8-16 cell (Sakkas, Batt & Cameron, 1989), cow 4-8 cell (Camous, Heyman, Meziou & Menezo, 1984) and pig 4-cell (Davis, 1985).

For many of these species, the stage of developmental arrest coincides with certain other developmental transitions, including the activation of transcription by the embryonic genome (Bolton, Oades & Johnson, 1984; Sakkas *et al* 1988; Brade *et al* 1988), the selective inactivation or destruction of much of the pre-existing maternal mRNA (Bolton *et al* 1984; Paynton, Rompel & Bachvarova, 1988), and a transition from extended to shorter cell cycles (Smith & Johnson, 1986; Crosby, Gandolfi & Moor, 1988).

In the mouse, 1-cell zygotes from most outbred and inbred strains do not develop to blastocysts when cultured in a chemically defined medium, but arrest during cleavage, the so-called "2-cell block" (Whittingham, 1974).

In contrast, embryos from F1 crosses between certain inbred strains can develop into normal blastocysts in the same

culture medium. Whether or not the embryos block during cleavage depends on the genotype of the egg (Goddard & Pratt, 1983).

The block is evidently caused by a cytoplasmic deficiency, since embryos that block can be rescued by the transfer of cytoplasm from a non-blocking strain embryo at the G1 or G2 phase of the cell cycle (Muggleton-Harris *et al*, 1982; Pratt & Muggleton-Harris, 1988). No specific agent has been identified.

We have now discovered that the period over which development of mouse embryos becomes blocked is characterised by a transient rise in the embryonic level of reactive oxygen species such as $H_2O_2$ (Nasr-Esfahani *et al*, 1990 copy filed herewith).

The presence of $H_2O_2$ and superoxide anions can give rise to the production of highly toxic hydroxy free radicals via the Haber-Weiss reaction (Halliwell & Gutteridge, 1985). Free radicals have been implicated in the prevention of cell division, in loss of cell function and in damage to cells in certain pathological conditions (Halliwell & Gutteridge, 1985; Halliwell, 1987; Aitken & Clarkson, 1987, 1988 & 1989). It therefore seemed possible that the generation of damaging free radicals from the elevated $H_2O_2$ detected at the mid to late 2-cell stage might be involved in the causation of the block.

However, 2-cell embryos from both blocking and non-blocking strains alike showed the transient rise in $H_2O_2$ levels. Thus, if free radicals are involved in the block of development then either there must be less conversion of $H_2O_2$ to free radicals in non-blocking than in blocking strains or any free radicals that are generated must be scavenged more efficiently in non-blocking than in blocking strains. The generation of free radicals by the Haber-Weiss reaction requires the presence of iron (Halliwell &

Gutteridge, 1985):

(1) $Fe^{3+} + O_2^{\cdot-} \rightarrow O_2 + Fe^{2+}$

(2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-}$

(3) Net reaction: $O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^{-}$

One consequence of free hydroxy radical generation is the peroxidation of polyunsaturated membrane lipids. Once peroxidation is initiated, free ferrous ions can themselves promote further peroxidation damage directly. Thus, iron is known to be potentially very damaging to development, as has been noted previously (Abramozuk *et al* 1977; Hoshi & Toyoda, 1985; Schini & Bavister, 1988; Toyoda *et al*, 1989).

According to the present invention, there is provided a culture medium for the culture of mammalian embryos wherein the culture medium contains at least one iron chelator.

The iron chelator may be any compound capable of binding to iron without adversely affecting replication of the embryo.

Preferably, the iron chelator is a ferroxidase. That is, an oxidising agent capable of oxidising Fe(II) to Fe(III). Such chelators have the advantage of inhibiting hydroxy radical formation by depriving the Haber-Weiss reaction of Fe(II).

Preferably, the iron chelator is capable of binding other cations, such as copper cations. This again inhibits hydroxyl radical formation.

Preferably, the iron chelator does not bind iron so strongly as to deprive the culture media of catalytic amounts of iron essential for embryo development.

Bovine apotransferrin is particularly preferred, but the iron chelator may be ethylenediaminetetracetic acid (EDTA)

or diethylenetriaminepentacetic acid (DETAPAC).

The culture medium may contain nutrients and other reagents as known in the art.

The culture medium preferably has a concentration of the iron chelator from 0.05 to 2 mg/ml, preferably 0.1 to 1.5 mg/ml, most preferably about 1 mg/ml, for bovine apotransferrin. These concentrations being especially suitable for mouse embryo culture.

The culture medium preferably has a concentration of the iron chelator of from 0.05 mM to 0.5 mM, most preferably 0.1 mM to 0.2 mM for DETAPAC. These concentrations being especially suitable for mouse embryo culture.

The culture medium preferably has a concentration of the iron chelator of from 0.05 mM to 0.2 mM, most preferably from 0.1 to 0.15 mM for EDTA. These concentrations being especially suitable for mouse or hamster embryo culture.

According to a second aspect of the invention, there is provided a method of *in vitro* mammalian embryo culture comprising culturing an embryo in a culture medium comprising an iron chelator at least in the period between fertilisation and the formation of a blastocyst.

The mammalian embryo is preferably an embryo resulting from an *in vitro* fertilisation step. The embryo may be the embryo of a human or non-human mammal.

The invention is now described in detail, by way of example only.

# 1. Materials and Methods

## 1.1. Embryo Handling

MF1 female mice (3-4 weeks; Central Animal Services, Cambridge, UK) and F1 female mice (C57BL/10ScSn/Ola female x CBA/Ca/Ola male, bred in the laboratory) were superovulated by intraperitoneal injection of 5 to 10 iu of pregnant mare's serum gonadotrophin (PMS; Intervet, Cambridge, UK) at midday followed 48 hours later by 5 or 10 iu of human chorionic gonadotrophin (hCG; Intervet). To obtain 1-cell fertilized zygotes, females were paired individually overnight with HC-CFLP males (Interfauna, Wyton-Huntingdon, UK) and inspected for vaginal plugs the next day as an indication of successful mating. Fertilized eggs at the pronuclear stage were recovered from mated females at between 26 and 30 hours post-hCG by release from the oviduct into warmed medium H6+BSA (a modified form of T6 medium, Howlett *et al*, 1987).

In a preliminary series of experiments, two culture media (M16 and T6; compositions recorded in Table 1) were compared in parallel for their effectiveness in permitting 1-cell zygotes of both blocking and non-blocking strains to develop to blastocysts.

Table 1: Composition of the media T6, H6 and M16

| Component | T6 | H6 | M16 |
|---|---|---|---|
| | | mM | |
| NaCl | 80.77 | 80.77 | 94.63 |
| KCl | 1.84 | 1.84 | 4.83 |
| $NaH_2PO_4 \cdot 2H_2O$ | 0.39 | 0.39 | 0.00 |
| $KH_2PO_4$ | 0.00 | 0.00 | 1.19 |
| $NaHCO_3$ | 25.00 | 4.10 | 25.00 |
| Sodium pyruvate | 0.27 | 0.27 | 0.33 |
| Sodium lactate | 23.30 | 23.30 | 23.30 |
| D-Glucose | 5.55 | 5.55 | 5.55 |
| $MgCl_2 \cdot 6H_2O$ | 0.49 | 0.49 | 0.00 |
| $MgSO_4 \cdot 7H_2O$ | 0.00 | 0.00 | 1.19 |
| $CaCl_2 \cdot 2H_2O$ | 1.77 | 1.77 | 1.71 |
| HEPES | 0.00 | 21.00 | 0.00 |
| | | mg $ml^{-1}$ | |
| Penicillin | 0.06 | 0.06 | 0.06 |
| Streptomycin | 0.05 | 0.05 | 0.05 |
| BSA | 4.00 | 4.00 | 4.00 |
| Phenol red | 0.01 | 0.00 | 0.00 |

(The osmolarity of the medium as made up is 255 mosmoles. This osmolarity is compatible with development, but we routinely adjunct to 280 mosmoles using 20% NaCl. The pH of H6 is adjusted to 7.4. The pH of M16 and T6 is adjusted to 7.6 before incubation in 5% $CO_2$, followed after equilibration with a check to confirm that the pH has reduced to 7.4.)

In all trials without exception, the modified medium T6 was found to be better. In all the experiments described herein, embryos were cultured in drops of T6+BSA under paraffin oil (FSA Laboratories, Loughborough, UK) in Falcon tissue culture dishes in 5% $CO_2$ in air. All manipulations were carried out at 37°C on heated stages, pads or in incubators.

Zygotes were cultured under the conditions specified in Section 2 and inspected at regular intervals as follows: at 47-50 hours post-hCG the few 1-cell or abnormal eggs were

removed and are not included in the totals, the remaining embryos being scored as 2-, 3- or 4-cells; at 69-72 hours post hCG embryos were scored as being dead, 2-cell, 3-cell, 4-cell, 5- to 8-cell precompact, or compact; at 98-100 hours post-hCH embryos were scored as being dead, noncompact, compact, early blastocyst or expanded blastocyst (see Chisholm *et al*, 1985, for definitions of blastocyst subtypes); at 116-119 hours post-hCG embryos were scored as being dead, preblastocyst, early blastocyst or expanded blastocyst.

Injection of catalase into 1-cell zygotes was done on a Leitz micromanipulator using rhodamine dextran as an injection marker as described in detail in McConnell *et al* (1990).

For transfer of blastocysts to the uteri of pseudopregnant females, recipients were used 2.5 days after being plug positive with vasectomized male. The transfer technique used was as described in Hogan *et al*, (1986). Females were autspoied at 10.5 days of pregnancy. Any females with no implantation sites were not included in the results.

1.2. Chemicals

Additives to the culture medium were obtained as follows: Sigma (Poole, UK): Bovine transferrin (iron free); human transferrin (iron free); $FeSO_4$; ascorbic acid. CIBA (Horsham, UK): Desferrioxamine B methansulphone (Desferal); Diethylenetriaminepentaacetic acid $(DETAPAC)_4$. Calbiochem (Cambridge, UK): catalase. After making stock solutions of additives in culture media, the pH of the medium was measured and, if necessary adjusted, to bring it to pH 7.4 under 5% $CO_2$.

Iron loading of transferrin was done according to the procedure of Farb & Frieden (1979). Bovine iron free transferrin (10 mg) was added to 2 ml of freshly prepared

bicarbonate solution (100 mM) containing ferric chloride at various concentrations (0-25 mM) for 30 minutes at pH 8.8. The pH of each solution was then adjusted to 7.4 and the absorbance of each solution was read at 470 nm. The plot of ferric chloride concentration against absorbance showed that the apotransferrin was fully saturated with $Fe^{3+}$ at the highest ferric chloride concentrations. Then the iron loaded transferrin was dialysed against T6 before use.

## 2. Results

2.1. We have discovered that the addition of exogenous iron to the culture medium impairs the development of embryos *in vitro*. F1 embryos were incubated in $FeSO_4$ and ascorbate (to maintain the iron in a ferrous state) in a ratio of 1:5 over a range of iron concentrations. The results are shown in Table 2 (experiments 2-4) and reveal that development is affected adversely, the embryos blocking at early cleavage, many of them dying subsequently.

Table 2: Effect of free iron on development *in vitro* of 1-cell mouse zygotes to the blastocyst stage

| Culture condition | Percentage of zygotes forming blastocysts by 118h post-hCG (number of zygotes studied) | | | |
|---|---|---|---|---|
| | Exp.1 | Exp.2 | Exp.3 | Exp.4 |
| | MF1 | F1 | F1 | F1 |
| T6 only | 24(112) | 75(39) | 93(45) | 90(82) |
| T6+transferrin | 57(112) | 100(30) | 100(18) | 99(86) |
| FeSO4(μM):ascorbate(μM)* | | | | |
| 12.5:62.5 | ND | 64(22) | ND | ND |
| 25:125 | 14(72) | 40(40) | 90(14) | 49(79) |
| 50:250 | 1(74) | 28(47) | 84(19) | 53(74) |
| 100:500 | 7(73) | 0(30) | 80(15) | 0(79) |
| 200:1000 | 5(57) | 0(7) | 75(20) | 0(23) |

* In the experiments using MF1 embryos, transferrin was also present in the iron ascorbate media

2.2. We examined whether the natural iron chelator, transferrin could prevent the two-cell block. One-cell MF1 (blocking) zygotes were recovered from the oviducts and cultured in medium T6+BSA containing bovine apotransferrin over a range of concentrations from 0.01 to 4 mg $ml^{-1}$. The results from three typical experiments are shown in Table 3.

Table 3: Effect of incubation in iron-free bovine transferrin on the development of MF1 1-cell zygotes *in vitro*

| [Apo-Transferrin] mg/ml | Number of embryos | % embryos at 70 hours >3-cells | % embryos at 70 hours Compact | % embryos at 96 hours blastocysts | % embryos at 118 hours blastocysts |
|---|---|---|---|---|---|
| 0 | 47 | 77 | 6 | 4 | 28 |
| 0 | 57 | 18 | 2 | 0 | 2 |
| 0 | 93 | 56 | 3 | 10 | 11 |
| 0 | 117 | 62 | 3 | 0 | 18 |
| 0.01 | 68 | 76 | 13 | 12 | 27 |
| 0.01 | 122 | 50 | 1 | 2 | 13 |
| 0.05 | 116 | 90 | 5 | 9 | 32 |
| 0.1 | 112 | 96 | 19 | 55 | 73 |
| 0.1 | 46 | 93 | 39 | 57 | 87 |
| 0.5 | 44 | 95 | 30 | 59 | 91 |
| 1 | 139 | 96 | 50 | 68 | 94 |
| 1 | 56 | 95 | 57 | 52 | 96 |
| 1 | 110 | 94 | 14 | 36 | 89 |
| 1.5 | 40 | 95 | 40 | 50 | 78 |
| 2 | 48 | 88 | 52 | 56 | 81 |
| 4 | 27 | 70 | 19 | 0 | 15 |
| 4 | 65 | 46 | 4 | 47 | 47 |

In T6 medium alone, over 50% of MF1 embryos will divide to 4-cells, albeit with a considerable delay, and of these a variable number (up to 30%) will form blastocysts. However, the presence of apotransferrin in the T6 medium at concentrations between 0.1 and 2 mg $ml^{-1}$ is associated with a marked increase in the numbers of 4-cell and compact embryos at 70 hours and the number of blastocysts at 98 and 118 hours post-hCG. Lower concentrations of apotransferrin did not overcome the block, whilst at higher concentrations embryonic death was observed.

The presence of apotransferrin was not required throughout the period of culture *in vitro* to the blastocyst, since exposure to apotransferrin (1 mg $ml^{-1}$) immediately after recovery of zygotes from the oviduct (26 hours post-hCG at the late 1-cell stage) for a period of 22 hours (at which time transition to the 4-cell stage is occurring) was as effective in overcoming the block as continuous culture.

In an experiment to show this, 1-cell MF1 zygotes were recovered from the oviduct between 22 and 26 hours post-hCG and placed in culture until 118 hours post-hCG, when the incidence of blastocysts was noted.

The experiment showed that if the period of exposure of embryos to apotransferrin was reduced to less than 22 hours, fewer blastocysts developed. A reduced rate of development to blastocysts was also observed if culture in apotransferrin was maintained at a total of 22 hours but was either preceded immediately after recovery by a period of culture in apotransferrin-free T6 medium, or if recovery from the oviduct was advanced four hours earlier (to 22 hours post hCG) and embryos were removed from apotransferrin into T6 a corresponding four hours earlier at 44 hours post-hCG i.e. prior to division to the 4-cell stage.

Of 40 MF1 embryos grown to blastocysts in the continuous presence of apotransferrin and transferred to pseudopregnant

recipient mice, 38 implanted to form viable and apparently normal fetuses at 10.5 days of pregnancy. Blastocysts grown in T6+apotransferrin and maintained *in vitro*, expand and hatch.

The development *in vitro* of MF1 embryos in apotransferrin is not accompanied by a reduced level of reactive oxygen species such as $H_2O_2$ over the period of the 2- to 4-cell transition. We have shown this in an experiment in which freshly recovered MF1 fertilized oocytes (29 hours post-hCG) were cultured in T6+BSA or T6+BSA+apotransferrin in 1 mg $ml^{-1}$ and then were incubated for 15 minutes in DCHFDA ($1x10^{-5}$ M) at various times post hCG. Readings of DCF emission provide a measure of the level of reactive oxygen species (Nasr-Esfahani *et al* 1990 attached hereto).

Neither human apotransferrin (whether used fresh or after dialysis against T6 medium overnight) nor desferal overcame the block of development whether applied continuously (Table 4; data from two separate experiments shown) or as a pulse over the period of the 1-cell or 2-cell transition (data not shown).

Moreover, at higher doses of both these chelators, large numbers of embryos died.

However, both DETAPAC (0.1 to 0.2 mM) and to a lesser extent EDTA (0.01 mM) did permit further development, the latter rather variably (Table 4).

Table 4: Effect of incubation in various iron-chelators on the development of MF1 1-cell zygotes *in vitro*

| Iron Chelator | Number of embryos | % embryos at 70 hours >3-cells | % embryos at 70 hours Compact | % embryos at 96 hours blastocysts | % embryos at 118 hours blastocysts |
|---|---|---|---|---|---|
| **Human apo-transferrin** | | | | | |
| 1 mg/ml | 89 | 59 | 1 | 0 | 0 |
| *2 mg/ml | 79 | 86 | 0 | 0 | 0 |
| *4 mg/ml | 88 | 59 | 1 | 0 | 0 |
| **Desferal** | | | | | |
| 0.0001 μM | 97 | 46 | 1 | 3 | 14 |
| 0.001 μM | 126 | 46 | 0 | 1 | 10 |
| 0.005 μM | 29 | 68 | 0 | 0 | 4 |
| *0.01 μM | 148 | 45 | 0 | 0 | 3 |
| *0.1 μM | 119 | 0 | 0 | 0 | 0 |
| **Detapac** | | | | | |
| 0.01 mM | 54 | 46 | 0 | 0 | 7 |
| 0.05 mM | 56 | 80 | 2 | 5 | 30 |
| 0.1 mM | 137 | 92 | 18 | 34 | 80 |
| 0.2 mM | 60 | 98 | 23 | 32 | 82 |
| 0.5 mM | 58 | 88 | 12 | 22 | 59 |
| *1.0 mM | 84 | 37 | 0 | 2 | 0 |
| *10.0 mM | 98 | 0 | 0 | 0 | 0 |
| **EDTA** | | | | | |
| 0.01 mM | 39 | 0 | 0 | 0 | 0 |
| 0.05 mM | 37 | 1 | 0 | 0 | 2 |
| 0.1 mM | 168 | 81 | 0 | 1 | 34 |
| 0.1 mM | 41 | 90 | 0 | 15 | 58 |
| 0.2 mM | 47 | 23 | 0 | 0 | 9 |

* indicates a large amount of embryonic death in culture

In order to determine whether the positive effects of bovine apotransferrin depended on its capacity to chelate iron in culture, an iron saturated preparation of bovine transferrin was prepared. A second sample of apotransferrin was taken through the iron saturating protocol in the absence of iron as a control. Both samples were then compared for their ability to overcome the block relative to a non-treated sample of bovine apotransferrin.

In the experiment, MF1 1-cell zygotes were recovered at 26 to 29 hours post-hCG, cultured until 119 hours post-hCG in T6 medium alone or containing control apotransferrin at 1 mg/ml, or various concentrations of either iron-saturated transferrin or apo-transferrin taken through a mock iron loading procedure and the incidence of blastocyst formation recorded. The rate of blastocyst formation in apo-transferrin taken through a mock loading procedure is lower than that of control apo-transferrin at 1 mg $ml^{-1}$, suggesting that some of the transferrin is denatured during the loading procedure.

The results revealed that iron-saturated transferrin is as effective as apotransferrin in supporting development to the blastocyst stage.

In addition, when MF1 embryos were incubated in medium containing both apotransferrin and free iron, development through the 2-cell block did not occur (Table 2 above; experiment 1).

It will be understood that the invention is described above by way of example only and modifications of detail may be made within the scope of the present invention.

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**The relationship between the rise in $H_2O_2$ and the "2-cell block" to development is discussed.**

## Introduction

The culture of preimplantation mammalian embryos from fertilization to the blastocyst stage has not been achieved for most species. In all species studied, evidence has been found for arrest of development at a specific cell stage, e.g. mouse 2-cell (Whittingham, 1974; Goddard & Pratt, 1983), human 4-8 cell (Braude *et al.* 1988), hamster 2-cell (Carney & Bavister, 1987; Bavister, 1988), sheep and goat 8-16 cell (Sakkas *et al.* 1989), cow 4-8 cell (Camous *et al.* 1984) and pig 4-cell (Davis, 1985). For many of these species, the stage of developmental arrest coincides with certain other developmental transitions, including the activation of transcription by the embryonic genome (Bolton *et al.* 1984; Sakkas *et al.* 1988; Braude *et al.* 1988), the selective inactivation or destruction of much of the pre-existing maternal mRNA (Bolton *et al.* 1984 ; Paynton *et al.* 1988), and a transition from extended to shorter cell cycles (Smith & Johnson, 1986; Crosby *et al.* 1988).

The phenomenon of developmental arrest has been studied most extensively in the mouse, in which 1-cell embryos from most outbred and inbred strains do not develop to blastocysts when cultured in a chemically defined medium, but arrest at the 2-cell stage, a phenomenon referred to as the " 2-cell block" (Goddard & Pratt, 1983). In contrast, embryos from certain inbred strain crosses can develop into normal blastocysts in the same culture medium. Studies involving reciprocal crosses between different strains of mice suggest that the genotype of the oocyte alone determines whether the embryos block at the 2-cell stage (Goddard & Pratt, 1983; Loutradis *et al.* 1987). The 2-cell block can be overcome by the transfer of a small amount of cytoplasm from a non-blocking strain in the $G_1$ or $G_2$ phase of the second cell cycle to the embryos of a blocking strain. It has been proposed that a specific factor(s) might be absent in blocking strains as the result of a deficiency in the *in vitro* environment (Muggleton-Harris *et al.* 1982; Pratt & Muggleton-Harris, 1988; Muggleton-Harris & Brown, 1988), but no specific agent has been identified. Although the mechanism(s) underlying the 2-cell block in mice is not yet established, it is clear from the studies of cell lines *in vitro* that oxidative species including hydrogen peroxide and free radicals (ie superoxide anions and hydroxy radicals) can be involved in damaging cells. These reactive oxygen species have also been implicated in the damage seen in certain pathological conditions associated with the arrest of cell division and loss of cell function (Halliwell & Gutteridge,

1989; Halliwell, 1987; Aitken & Clarkson, 1987, 1988; Aitken *et al.* 1989). It is possible therefore that such reactive oxygen species might also be involved in the generation of the 2-cell block. In order to examine this hypothesis, we have developed a technique for assessing the level of $H_2O_2$ in individual oocytes and cleavage stage embryos.

To quantify $H_2O_2$ in individual oocytes and embryos, we have modified an assay for $H_2O_2$ in aqueous solution (Keston & Brandt, 1965; Brandt & Keston, 1965) for use by photocytometry. 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) and 5(and 6)-carboxy-2,7, dichlorofluorescein diacetate (CDCFDA) are related compounds which, because of their non-ionized state, are membrane permeant and therefore are able to diffuse readily into cells. Within the cell, the acetate groups are hydrolyzed by intracellular esterase activity forming 2',7'-dichlorodihydrofluorescein (DCHF) and 5(and 6)carboxy-2,7,dichlorofluorescein (CDCF) which are polar and thus trapped within the cell. CDCF is fluorescent at intracellular pH and provides a measure of the uptake and hydrolysis of the dyes. However, DCHF fluoresces only after oxidation by $H_2O_2$ to yield 2',7'-dichlorofluorescein (DCF). The level of DCF produced within the cells appears to be related linearly to that of $H_2O_2$ present (Bass *et al.* 1983, 1986; Cathcart *et al.* 1983) and thus its fluorescent emission provides a measure of $H_2O_2$ levels. We have applied this technique to embryos from two strains of mice: MF1 (a blocking strain) and F1 (a non-blocking strain) over the period during which the 2-cell block develops. Freshly recovered oocytes and embryos have been compared with those cultured *in vitro* for varying times.

## Materials and methods

MF1 female mice (3-4 weeks; Central Biological Services, Cambridge, UK) and F1 female mice (C57BL/10ScSn/Ola female x CBA/Ca/Ola male, bred in the laboratory) were superovulated by intraperitoneal injection of 5 or 10 i.u. of pregnant mare's serum gonadotrophin (PMS; Intervet) and human chorionic gonadotrophin (hCG; Intervet) 48 h apart. To obtain embryos, females were paired individually over night with HC-CFLP males (Interfauna) and inspected for vaginal plugs the next day as an indication of successful mating.

Unfertilized oocytes were recovered from unmated females at about 13 h post-hCG. 2-, 4- and 8-cell embryos were recovered at 36-48, 50-54 and 69-70 h post-hCG respectively. Oocytes and embryos were released from the oviduct into warmed

H6+BSA (a Hepes buffered variant of T6), and then cultured in drops of T6+BSA (Howlett *et al.* 1987) under paraffin oil (Martindale), in Falcon tissue culture dishes, in 5% $CO_2$ in air. All manipulations were carried out at 37°C on heated stages, pads or in incubators. Microinjection of 2-cell embryos was carried out as described in McConnell *et al.* (1990).

A stock solution of $H_2O_2$ (30% weight/volume) was obtained from BDH plc (Poole; England). Bovine liver catalase was obtained in lyophilized form from Calbiochem (Cambridge Bioscience, UK).

Stock solutions of DCHFDA (Kodak, Eastman Kodak Company, Rochester, NY, USA.) and CDCFDA (Molecular Probes Inc, Pitchford Ave, Eugene, USA) were prepared in acetone at $1\times 10^{-3}$ M. The stock solutions were diluted in H6+PVP to the required concentration. The solutions of CDCFDA were prepared freshly before use, since CDCFDA becomes de-natured on exposure to air. DCHFDA stock solutions were also prepared just before the start of each experiment and were kept in the dark and used over a maximum period of 48 hours. Oocytes and embryos were washed thoroughly in H6+PVP after their removal from H6 or T6+BSA and then loaded with the dyes in cavity blocks for a specified time (generally 15 minutes). The oocytes or embryos were then washed in H6+BSA to remove traces of the dyes and were placed in specially designed small chambers containing H6+BSA and covered by a coverslip (Maro *et al.* 1984). The fluorescence emissions of the oocytes and embryos were measured immediately by photocytometry, using a perspex carrying slide for viewing with a long working distance × 32 objective on a Leitz Ortholux II microscope with stabilized HBO100 mercury vapor lamp and filter set L1 for FITC. For quantification of fluorescence, the photomultiplier housing of a Leitz MPV-1 was fitted to the Ortholux II phototube (McConnell *et al.* 1989). The phototube contained a variable measuring diaphragm that could be adjusted to surround the periphery of an individual oocyte or embryo, thus excluding background. A 6.25% transmission neutral density filter (Leitz N16) was placed in the path of the exciting light, to minimise any potential damage to the cells. Fluorescent emission was deflected to the amplifier/discriminator (Model 1140B, SSR Instruments Co, USA) of a quantum photometer (Model 1140A, SSR Instruments) that had been zeroed against background and set to read in a counts $s^{-1}$ mode via a deflection meter. The individual oocyte or embryo was positioned within the adjusted diaphragm and exposed to the excitation wavelength for a period of less than 10 s and the fluorescent emission recorded (counts $s^{-1}$ on the 1M scale to a maximum reading of 10). This set up, involving short exposure to the

exciting light was designed to avoid damage to the oocytes or embryos, which was only detected with greater than a 40 s exposure to the exciting light or removal of the 6.25% transmission neutral density filter. Under these latter conditions, a rapid rise in fluorescent emission, reflecting conversion of DCHF to DCF, was detected, probably as a secondary consequence of lipid peroxidation. For each data point in each experiment, the fluorescent emissions of 10 to 20 oocytes or embryos were measured and their mean values were expressed as the 'mean reading'.

## Results

### *A technique for measuring $H_2O_2$ in individual mouse embryos*

Experiments were designed to observe whether we could measure a fluorescence signal from DCF or CDCF in single embryos. Within 1h of their removal from the oviducts, freshly recovered oocytes and embryos were incubated for 15 minutes in the dyes, washed and their fluorescent emission measured immediately. Fig. 1 shows that the level of CDCF fluorescence remains constant from the unfertilized oocyte to the 8-cell stage. This result suggests that the uptake and de-esterification of the dyes does not vary with developmental stage. In contrast, the fluorescence from DCF is much lower and appears to increase slightly between the unfertilized oocyte and 8-cell stages, the biggest change appearing during the transition from the 1- to 2-cell stage, but even then does not approach the level of fluorescence generated by incubation in equimolar concentrations of CDCFDA. Since any fluorescence deriving from incubation in DCHFDA requires an oxidative step, these results suggest (a) that such $H_2O_2$ as is produced is insufficient to oxidize all the DCHF generated by esterase activity, (b) that the level of $H_2O_2$ may increase slightly with the more advanced developmental stages, and (c) that the increase observed is not due simply to an increased rate of uptake and/or hydrolysis of the dyes.

Kinetic studies of (i) the rate of dye uptake and conversion and (ii) the rate of decay of fluorescent signal after a loading period followed by transfer to washing medium were carried out on unfertilized oocytes, fertilized oocytes and 2-cell embryos of both MF1 and F1 strains of mice. Fig. 2 (panels A-C) show typical plots obtained from kinetic studies of the three different cell stages with embryos incubated in CDCFDA or DCHFDA. The 'increase in reading' from the fluorescent derivatives of CDCFDA and DCHFDA remains approximately the same at each of the three different stages. However, the rise in signal after incubation in DCHFDA is much slower than that seen from oocytes and embryos incubated in one tenth the concentration of

CDCFDA because of the requirement for oxidation of the DCFH. In contrast, the decay of signals for both dyes is less after the transition from the 1- to 2-cell stage, a result that probably reflects reduced leakage of the dyes from the cells. This result might suggest that the apparent slight rise in DCF levels between the 1- and 2-cell stages described earlier (Fig. 1) could be due simply to a decrease in decay rate at the 2-cell stage. However, there are two reasons why this cannot be the case. First, examination of the fluorescent signal resulting from incubation in CDCFDA reveals similar kinetic decay patterns to those seen after incubation in DCHFDA, but no increase in fluorescence from CDCF occurs during the transition from the 1- to 2-cell stage. Second, all experimental fluorescence readings are taken after a 15 minute incubation in dye and within 5 minutes of removal from the dye, in which time the decay of signal does not differ between stages. Thus, the increased fluorescence emission from DCF at the 2-cell stage cannot be explained simply on the basis of greater retention of the fluorochrome, but must also involve greater production of DCF as a result of oxidation by $H_2O_2$ (see also below).

In order to establish further that our method was providing a quantitative measure of $H_2O_2$ levels in mouse embryos, we tried to enhance DCF formation from DCFH either by the addition of exogenous $H_2O_2$ or by lipid-peroxidation using high levels of UV radiation which increase intracellular peroxide levels. The results in Table 1 show that the DCF formation is increased if an extracellular threshold level of $H_2O_2$ greater than 0.0003% by volume is achieved or if lipid peroxidation is induced. Moreover, the signal from DCF under such conditions approximates to that of CDCF at the same concentration. Addition of catalase blocked the increased DCF formation induced by exogenous $H_2O_2$ but not that intrinsic to the cell or induced by UV radiation (Table 2). In contrast, endogenous DCF formation was reduced significantly (paired student t-test; $P<0.02$) if catalase was injected into one blastomere prior to incubation in DCHFDA (Table 2).

*Comparison of $H_2O_2$ production in vitro and in vivo*

Since DCHFDA appears to provide a valid method for the quantitative analysis of $H_2O_2$ production in individual mouse embryos at different times over the early cell cycles, we compared the fluorescent emission of embryos developed *in vitro* with those developed *in vivo* in two strains of mice, only one of which (MF1) shows the 2-cell block. Figs. 3 and 4 show (a) that, as suggested earlier, there may be a slight rise in DCF formation when freshly recovered 2-cell embryos are compared with those freshly recovered at 1-cell stage; and (b) that in both MF1 (Fig. 3) and F1 (Fig. 4) embryos the fluorescence emission from DCF increases markedly during G2 of the

second cell cycle when embryos are cultured *in vitro* but not when they have developed *in vivo*.The DCF fluorescence from *in vitro* cultured embryos declines during late G2/M (or its equivalent in developmental time in blocking embryos), and is restored to background levels on entry into the 4-cell stage. When embryos were incubated *in vitro* for a longer period through the third mitotic division into the 8-cell stage, no equivalent marked rise in DCF formation occurred (Fig. 5 shows the data for F1 embryos but the same result has been obtained with MF1 embryos - data not shown). The higher DCF levels after *in vitro* culture are due to an increase in the rate of its formation from DCHF oxidation (compare panels C and D in Fig. 2). In contrast to the results with DCHFDA, fluorescence from embryos incubated in CDCFDA is similar throughout whether they have developed *in vitro* or *in vivo*, indicating that differences in uptake, de-esterification and decay of the dyes cannot be responsible for the change in DCF fluorescence described above (Fig. 6 and compare panels C and D in Fig. 2).

To determine whether the absolute time spent *in vitro* influences the pattern of DCF fluorescence emission, embryos were cultured for different periods *in vitro* but were assayed at the same time e.g. 45-47 hours post-hCG (Fig. 7). The results from this experiment show that, regardless of the time spent *in vitro*, the $H_2O_2$ levels are elevated by 45-47 hours, corresponding to the late G2 period of the second cell cycle. Paradoxically, in the experiments recorded here some of those embryos that had spent the longest time *in vitro* (21-22 hours) showed the least marked rise in $H_2O_2$ production. This may reflect the fact that their development had been slowed and they were reaching the G2 period of the second cell cycle slightly later than the other groups. In confirmation of this, we have shown in subsequent experiments that the $H_2O_2$ levels in these embryos rose to maximal 2-3 hours later than those in the other two groups.

## Discussion

Hydrogen peroxide has been proposed as a second messenger in various cell stimulation and regulation systems (May & deHaen, 1979; Oberley *et al.* 1981; Skoglund *et al.* 1988; Laloraya *et al.* 1988, 1989), but may also, if the regulation of its levels is uncontrolled, contribute to cell damage by excessive peroxidation of lipids and proteins. In this paper, we describe a method for quantifying the production of reactive oxygen species in individual cells or embryos, thereby allowing us to examine the changes in these species over the early period of development.

The results presented here suggest that the amount of fluorescence emission from individual mouse oocytes and embryos is related to the rate of oxidation of DCHF by $H_2O_2$ produced within the oocytes and embryos. Thus, the fluorescence emission of DCHF but not that of CDCF can be enhanced by exogenous $H_2O_2$ or via lipid-peroxidation induced by irradiation with UV light. The signal from DCHF oxidation under such conditions approximates to that of CDCF. These results with single cells and embryos resemble those obtained fluorimetrically by Bass *et al.* (1986) using suspensions of polymorphonuclear neutrophilic leukocytes (PMNL). These workers showed that the rate of DCF formation was related linearly to that of $H_2O_2$ production, that the increase in the rate of DCF formation after activation of PMNL could be mimicked by addition of extracellular $H_2O_2$ or an $H_2O_2$ generating system (e.g. xanthine-oxidase + acetaldehyde), and that the increase seen with exogenous stimulation was inhibited by catalase but not by superoxide dismutase (SOD). We also were able to inhibit the oxidising effect of exogenous $H_2O_2$ with catalase, and the $H_2O_2$ generated within the cell was reduced by intracellular injection of catalase. Thus, it seems reasonable to conclude that the changing levels of DCF formation reflect changes in $H_2O_2$ levels in oocytes and embryos.

Our preliminary results suggested that there was a gradual increase in $H_2O_2$ levels as embryos proceeded from the unfertilized stage toward the 8-cell stage. However, a more detailed analysis of $H_2O_2$ levels in embryos freshly recovered from mice over the first three cell cycles (*invivo* embryos) suggested that this increase may not be as marked as at first evident and could reflect, at least in part, the consequences of exposure of embryos to conditions *in vitro* during recovery and handling. Thus, when analysis of *in vivo*-derived embryos is conducted rapidly after their recovery (as shown in Figs. 3, 4), the rise in $H_2O_2$ levels on transition to the 2- and 4-cell stages is much reduced. In contrast, when embryos spend a period *in vitro* before being incubated with fluorochrome, a marked rise in the fluorescent signal occurs, reflecting increased conversion of DCHF to DCF by oxidation. However, this increase in oxidative conversion *in vitro* is restricted to the G2/M phase of the second cell cycle, but is not evident before this time, nor after entry to the 4-cell stage is completed, nor during passage through the 4-cell stage and into the 8-cell stage. This temporal restriction on DCF formation is not a consequence of the total time spent *in vitro*, but reflects an interaction between *in vitro* conditions and the stage

of the cell cycle. The possibility that this increase was due simply to a greater retention of DCF was excluded by comparing the kinetics of handling of the two dyes by the embryos.

This increase in $H_2O_2$ production *in vitro*, compared with *in vivo* derived embryos, takes place during the atypically long G2 period of the second cell cycle, shortly after the time at which embryonic gene activation has occurred. This period coincides with that of the 2-cell block, suggesting a possible relationship between the block and the potential rise in damaging free radicals generated from the $H_2O_2$. Indeed, it has been proposed that activated oxygen species may arrest normal cell division (Oberley *et al*, 1981). However, when F1 embryos, which did not exhibit a 2-cell block *in vitro*, were compared with MF1 embryos, which did, both showed a rise in peroxide production in the latter half of the second developmental cell cycle. Thus, the absence of reactive oxygen species as a source of damaging free radicals cannot provide the simple explanation for the development of non-blocking strain embryos *in vitro*. However, it is possible that the MF1 embryos, unlike the F1 embryos, are less able to scavenge the reactive oxygen species induced by *in vitro* culture. The inability of MF1 embryos to develop normally *in vitro* might be due to a deficiency of cytoplasmic protective enzyme activity, such as is provided by superoxide dismutase, catalase or the glutathione peroxide/reductase couple.

In the context of these results, it is of interest that the development of preimplantation mouse embryos *in vitro* is sensitive to oxygen concentration, raising the possibility that oxygen toxicity might lead to developmental arrest via formation of oxygen radicals (Whitten, 1971; Quinn & Harlow, 1978; Pabon *et al.* 1989). Nonphysiological oxygen concentrations can lead to free radical generation via interaction with a number of cellular or media components, including hypoxanthine (Loutradis *et al* .1987), catecholamines ( Misra & Fridovich, 1972), thiols (Baccanari, 1978) and flavin (Ballou *et al.* 1969). An adverse effect of hypoxanthine on mouse embryo development was reported recently (Loutradis *et al.* 1987). Additionally, high oxygen tensions *in vitro* can influence the balance between the synthesis of glycogen (from exogenous glucose) and its degradation,

probably via accumulation of ATP (Ozias & Stern, 1973; Quinn & Wales, 1973; Barbehenn *et al.* 1974; Edirisinghe *et al.* 1984; Spielmann *et al.* 1984). These disturbances in glucose metabolism could lead to the formation of free radicals by generating NADPH via the pentose phosphate shunt, indeed glucose has been reported as deleterious for development through the 2-cell stage, its omission from the media improving development (Flood & Wiebold, 1988; Chatot *et al.* 1989). Finally, it may also be relevant that embryos blocked at the 2-cell stage show abnormalities in the organization of their newly synthesised membrane lipids and in their mitochondria (Muggleton-Harris & Brown, 1988; Pratt & George, 1989), both important targets for damage by free radicals.

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LEGENDS TO FIGURES

Fig. 1: Unfertilized MF1 oocytes and embryos were recovered; within one hour of recovery, they were incubated for 15 min in DCHFDA or CDCFDA at $1x10^{-5}$ M and the fluorescent emissions of DCF (hatched bar) and CDCF (solid bars) were measured immediately. S.D.s are shown. Unfer = unfertilized oocyte; Fer = fertilized oocyte; 2C, 4C and 8C = 2-, 4-, 8-cell stages.

Fig. 2: MF1 (A) unfertilized oocytes or (B) fertilized oocytes or (C) fresh 2-cell embryos or (D) in vitro aged 2-cell (cultured from 30-48 h post hCG) embryos were incubated with CDCFDA ( $5x10^{-6}$ M) or DCHFDA ( $1x10^{-5}$ M) for a period, the end of which is indicated by the astrisk (*). Then the embryos were washed in and transferred to H6+BSA and the amount of CDCF or DCF present was measured at different time points. Each point on the graph presents the mean reading for 10 embryos (± S.D.).

Fig. 3: Freshly recovered MF1 embryos (hatched bars) and MF1 embryos cultured *in vitro* from 23 h post-hCG (solid bars) were incubated for 15 minutes in DCHFDA ($1x10^{-5}$ M) at various times post hCG. Each bar represents the mean reading of DCF emission for 10 embryos plus S.D. Between 23 and 33 h post hCG all the readings were from 1-cell fertilized oocytes. Thereafter all the readings were from 2-cell embryos. Note that in non-blocking strains, embryos start dividing to 4-cells at shortly after 48 h post hCG (see Fig. 4) but do not do so in blocking strains such as MF1.

Fig. 4: Freshly recovered F1 embryos (hatched bars) and F1 embryos cultured *in vitro* (solid bars) were incubated for 15 min in DCHFDA ($1x10^{-5}$ M) at various times post hCG. Each bar represents the mean reading of DCF emission for 10 embryos plus S.D. Between 27 and 33 h post hCG all reading were from 1-cell fertilized oocytes. From 40 to 48 hours post hCG, all reading were from 2-cell embryos, thereafter only 4-cell embryos were read.

Fig. 5: F1 embryos were recovered at 43 hours post hCG and cultured *in vitro* for periods upto 60.5 hours post hCG. Samples of 10 embryos were taken at the times indicated, and incubated in DCHFDA at $1x10^{-5}$ M for 15 min. The mean values of DCF emission plus S.D. are shown. The reading are from 2-cell, 4-cell. or 5-8 cell embryos for each bar as indicated beneath.

Fig. 6: MF1 embryos were recovered and used immediately at 35 hours post hCG or cultured until 47 hours post hCG before use. Both sets of embryos were incubated for 15 min in CDCFDA or DCHFDA at $1x10^{-5}$M. Each bar represents the mean reading of CDCF (solid bars) or DCF (hatched bars) emission for 10 embryos plus S.D.

Fig. 7: MF1 and F1 embryos were recovered at various times post hCG, were cultured *in vitro* for 21-22 h, 15-16 h, 4-5 h or 0 h, and were then incubated for 15 min in DCHFDA ($1x10^{-5}$ M) at 45-47 h post hCG (which= time 0 h). Each bar represents the mean reading for 10-15 embryos. S.D. shown except in two bars (*) for which all readings were off scale in excess of 10.

Table 1. Effects of exogenous hydrogen peroxide or lipid peroxidation by UV irradiation on DCFand CDCF emission in fertilized MF1 oocytes.

| Treatment | Mean reading (In units) after inoubation in* DCHFDA (0.00001 M) | CDCFDA (0.00001M) |
|---|---|---|
| Control | 2.5(0.4) | 8.3 (1.2) |
| UV Irradiation | 9.0(0.7) | 8.3 (1.2) |
| Hydrogen peroxide: | | |
| 0.0003% | 2.1(0.6) | 8.8 (0.8) |
| 0.003% | 9.2(1.2) | 9.4 (0.7) |

* Values are mean for readings from 10 fertilized oocytes (plus S.D.).

Table 2. Fertilized MF1 oocytes were incubated in DCHFDA under various conditions and the emission from DCF recorded.

| Treatment | Mean reading (in units) after incubation in DCHFDA at 0.00001 M for 15 minutes |
|---|---|
| Fresh fertilized oocytes: | |
| Control | 1.4 (0.2) |
| Hydrogen peroxide (0.003%) | 10.0* |
| Hydrogen peroxide+catalase | 1.0 (0.1) |
| UV irradiation | 10.0* |
| UV irradiation+catalase | 10.0* |
| 2-cells: | |
| Fresh 2-cells | 2.4 (0.3) |
| 2-cells cultured from 28-42 h post hCG** | 9.2(0.6) |
| 2-cells cultured from 33-49 h post-hCG: | |
| Microinjected with water | 6.2 (0.7) |
| Microinjected with 0.5mg/ml catalase | 4.5 (0.4) |

Values are mean for the reading from 10 eggs (plus S.D.).
*The readings were off scale in excess of 10.
**Cultured in catalase for one hour before incubation in DCHFDA.

Claims

1. A culture medium for the culture of mammalian embryos wherein the culture medium contains at least one iron chelator.

2. A culture medium for the culture of mammalian embryo according to claim 1 wherein the iron chelator is bovine apotransferrin.

3. A culture medium for the culture of mammalian embryo according to claim 2 wherein the bovine apotransferrin has a concentration of from 0.05 to 2 mg/ml.

4. A culture medium for the culture of mammalian embryo according to claim 1 wherein the iron chelator is ethylenediaminetetracetic acid.

5. A culture medium for the culture of mammalian embryo according to claim 4 wherein the ethylenediaminetetracetic acid has a concentration of from 0.05 mM to 0.2 mM.

6. A culture medium for the culture of mammalian embryo according to claim 1 wherein the iron chelator is diethylenetriaminepentacetic acid.

7. A culture medium for the culture of mammalian embryo according to claim 6 wherein the diethylenetriamine-pentacetic acid has a concentration of from 0.05 mM to 0.5 mM.

8. A method of *in vitro* mammalian embryo culture comprising culturing an embryo in a culture medium comprising an iron chelator at least in the period between fertilisation and the formation of a blastocyst.

9. A method of in vitro mammalian embryo culture according to claim 8 wherein the mammalian embryo is an embryo resulting from an *in vitro* fertilisation step.

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